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TI The search for novel adjuvants for early life vaccinations: can "danger" motifs show us the way?.

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SO IMMUNOLOGIC RESEARCH, (1999) 19 (1) 35-46. Ref: 60

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Journal code: 8002742. ISSN: 0171-2985.

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TI DNA as an adjuvant

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TI Immunostimulatory DNA is a potent mucosal adjuvant

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Immunostimulatory Properties of Genomic DNA from Different Bacterial Species

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Abstract

Bacterial DNA has potent immunological properties because of its content of immunostimulatory sequences centering on CpG motifs. To investigate whether DNA from various bacterial species differ in these properties, the activity of a panel of DNA was assessed in *in vitro* cultures of murine spleen cells. This panel varied in base composition and included DNA from *Clostridium perfringens* (CP), *Escherichia coli* (EC), *Micrococcus lysodeikticus* (MC), *Staphylococcus aureus* (SA), and, as a mammalian DNA control, calf thymus (CT) DNA. In assays of IL-12 and IFN- γ production as well as B cell mitogenesis, these DNA showed marked differences in their immunostimulatory activity. For both cytokine and B cell responses, EC DNA demonstrated the highest activity while CP DNA had the lowest activity among the bacterial DNA. To determine whether differences in stimulatory capacity resulted from differences in cell uptake, the activity of DNA complexed with lipofectin was tested. While the addition of lipofectin to DNA increased stimulation by all DNA, it did not change the relative potency of the DNA tested. These results indicate that bacterial DNA differ in their immunostimulatory capacity, most likely reflecting their content of CpG motifs. These differences could affect the induction of innate immunity as well as the consequences of infection.

Introduction

DNA is an essential macromolecule whose immunologic properties vary with sequence heterogeneity. While mammalian DNA is immunologically inert, bacterial DNA has potent immunological properties. These properties include the induction of cytokines such as IL-6, IL-12, IFN- γ , IFN- α/β , and TNF- α , as well as stimulation of B cell mitogenesis (1–8). As shown using cloned DNA as well as synthetic oligonucleotides, these activities result from hexameric sequences that

Abbreviations: ISS = immunostimulatory sequence; IFN = interferon; TNF = tumor necrosis factor; MSR = macrophage scavenger receptor; LPS = lipopolysaccharide; dG = deoxyguanosine.

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cause of its content of immunostimulatory whether DNA from various bacterial DNA was assessed in *in vitro* cultures and included DNA from *Clostridium lysodeikticus* (MC), *Staphylococcus aureus* (CT) DNA. In assays of IL-12 and IFN- γ showed marked differences in their cell responses, EC DNA demonstrated activity among the bacterial DNA. To determine differences in cell uptake, the relative potency of the DNA tested. While the addition of lipofectin to DNA showed relative potency of the DNA tested. Their immunostimulatory capacity, most differences could affect the induction of

immunologic properties vary with DNA. A is immunologically inert, bacterial DNA. These properties include the induction of IFN- γ , IFN- α/β , and TNF- α , as well as using cloned DNA as well as DNA from hexameric sequences that

IFN = interferon; TNF = tumor necrosis factor; LPS = lipopolysaccharide; dG = deoxyguano-

center on an unmethylated CpG dinucleotide (2). These sequences, called CpG motifs or immunostimulatory sequences (ISS), have the structure of purine-purine-CpG-pyrimidine-pyrimidine (4). Because of differences in the pattern of base methylation as well as CpG suppression in mammalian DNA, ISS occur much more commonly in bacterial than mammalian DNA and may therefore function like endotoxin to stimulate innate immunity (9).

Recent studies of the immunostimulatory capacity of DNA have commonly used as models short oligonucleotides (oligos) containing ISS in the context of other sequences. While these oligos have activities that resemble those of bacterial DNA, the relationship between these compounds and natural DNA in their structure-function relationships and spectrum of immunostimulatory activities is unclear. Thus, some natural DNA fail to show properties predicted by their content of ISS. Unmethylated mammalian DNA, for example, is immunologically inactive despite its content of ISS (10). These findings have suggested that ISS may not be the sole determinant of immune stimulation by natural DNA.

Among DNA structures that could affect immune stimulation, sequences other than CpG motifs may act alone or in concert with ISS. Thus, extended runs of deoxyguanosine (dG) residues can increase stimulation of NK cell activity and IFN production by an ISS (11). This enhancement most likely results from increased DNA uptake into cells by the Type A macrophage scavenger receptor (MSR); this receptor binds dG-rich DNA among other polyanion ligands (12, 13). Furthermore, while dG sequences alone are unable to induce macrophage cytokine production, they can directly stimulate B cell mitogenesis (14). The manner in which various sequences function in high molecular weight natural DNA is unknown, although key to understanding the role of bacterial DNA in the setting of host defense.

To elucidate further immune stimulation by bacterial DNA and its relationship to base sequence, we have investigated the *in vitro* activity of a panel of bacterial and mammalian DNA. This panel included DNA from four bacterial species, *Clostridium perfringens* (CP), *Escherichia coli* (EC), *Micrococcus lysodeikticus* (MC), and *Staphylococcus aureus* (SA) as well as calf thymus (CT) DNA as a mammalian DNA control. These DNA differ in GC content as well as predicted content of CpG motifs. Using *in vitro* assays of cytokine production and B cell mitogenesis to assess immunostimulation, we show that these DNA differ markedly in their potency, with EC DNA displaying the highest, and, CP DNA, the lowest immunostimulatory activity. These results indicate the immunostimulation by bacterial DNA varies by species and suggest that the potency of bacterial DNA could represent a pathogen-derived factor influencing host defense and the outcome of infection.

Materials and Methods

Nucleic acids

DNA in these experiments were either commercially purchased (Sigma Chemical Co., St. Louis, MO, USA) or derived from organisms grown in broth culture. Purification of DNA from cul-

tures was performed with a resin-based extraction system (Qiagen, Chatsworth, CA, USA) designed to generate high molecular weight genomic DNA. Commercially purchased DNA were additionally purified by phenol extraction followed by ethanol precipitation. To verify that all DNA preparations were of comparable size, gel electrophoresis was performed by running 1 µg of DNA on 0.5% agarose gel at 25 V for 8 hr.

All DNAs tested were assayed for endotoxin content using the limulus amoebocyte lysis (LAL) assay (Bio-Whittaker, Walkersville, MD, USA). On the basis of these determinations, some DNA preparation were additionally treated by polymyxin affinity chromatography (Sigma) to remove any residual contamination. For all DNA preparations, the endotoxin concentration was less than 2.5 µg/mg DNA. This value represents the lower limit of detection of the LAL kit.

Prior to use, all nucleic acids were made single stranded by boiling for 15 min, followed by rapid cooling. For DNase experiments, DNA preparations were digested with 50 µg/ml DNase 1 (Sigma) for 2 hours in 10 mM MgCl₂, 50 mM Tris pH 7.5 (Sigma). The DNA were then heat denatured for use in culture. For experiments using cationic liposomes, complexes were made by adding Lipofectin reagent (Gibco/BRL, Gaithersburg, MD, USA) to DNA at 2:1 w:w ratio in serum free media for 15 min prior to addition to culture. The optimal ratio of lipofectin to DNA was derived from preliminary titration experiments in which various concentrations of this reagent were added to a constant amount of EC DNA.

Cell culture

Single cell suspensions were prepared from spleens obtained from 6–8 week old BALB/c or C3H/He J mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To assess immune activation, cells were cultured in RPMI 1640 media (Gibco/BRL) supplemented with 5% heat inactivated fetal bovine serum for 24 to 48 hours at 37 °C and 5% CO₂ in 96 well tissue culture plates (Costar, Cambridge, MA, USA). For assays of proliferation, CD69 expression, and IL-12 production, cells were cultured at 5×10^6 cells/ml. For assays of IFN-γ production, cells were cultured at 1×10^7 cells/ml. The DNA concentration in cell cultures ranged from 0.05–50 µg/ml. For all experiments performed, stimulation with LPS or concanavalin A was used as a control. All assays were performed in triplicate.

Thymidine incorporation

After 24 to 48 hours of stimulation, 0.5 µCi/well 3H-thymidine, 6.70 Ci/mmol (NEN, Boston, MA, USA) was added to 200 µL cell culture. After 7 hours of labeling, cells were collected onto glass fiber filters and radioactivity was measured by liquid scintillation counting. Additional experiments were performed to assess mitogenesis by incorporation of uridine using 0.5 µCi/well 3H-uridine, 38.4 Ci/mmol (NEN). This determination was done to ensure that proliferation assays using thymidine were not affected by label dilution from thymidine released by DNA broken down in culture (15). The result of these experiments showed the same rank order of stimulation. A separate set of control experiments was performed using endotoxin resistant C3H/HeJ mice to ensure that any endotoxin in the DNA preparations did not contribute to the pattern of stimulation observed.

CD69 expression

To assess cell activation by surface CD69 expression, cells were washed and suspended in FAC-Scan saline after 24 hours stimulation. Cells were labeled with PE- and FITC-conjugated anti-CD19 and anti-CD69 mAb (PharMingen, San Diego, CA, USA) according to the manufacturer's specifications. Mean fluorescence for CD69 was determined for both CD19⁺ (B cell) and CD19⁻ (non-B cell) populations using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). For each measurement, 10,000 events were collected.

Cytokine production

IL-12 and IFN-γ production with ELISA system (PharMingen). IL-12 were directed at the diluted 1:10 in phosphate buffered 0.05% Tween 20 (Sigma) by Dynatech, Chantilly, VA, USA.

Results

To evaluate differences in capacity, murine spleen DNA from 4 species were assayed. These DNA were used to assess cytokine production. We have shown that this DNA for *in vitro* stimulation but not mammalian DNA induced the response varied. MC DNA induced the active among the bacterial DNA at the highest concentration.

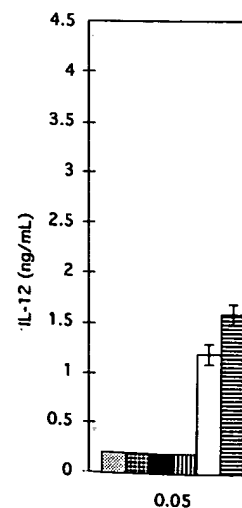


Figure 1. Dose response for IL-12 production for 24 hours with bacterial DNA by ELISA. Results are presented as mean ± SD of a representative experiment which was repeated three times.

Cytokine production

IL-12 and IFN- γ production was measured in supernatants at either 24 or 48 hours using a sandwich ELISA system (PharMingen) according to the manufacturer's directions. Antibodies for IL-12 were directed at the p40 subunit of the biologically active dimer. Supernatants were diluted 1:10 in phosphate buffered saline containing 0.5% bovine serum albumin (Sigma), and 0.05% Tween 20 (Sigma) before use. ELISAs were performed on Immulon-2 96-well plates (Dynatech, Chantilly, VA, USA).

Results

To evaluate differences among bacterial DNA in their immunostimulatory capacity, murine spleen cells were stimulated *in vitro* with highly purified bacterial DNA from 4 species; both cytokine production and mitogenesis were measured. These DNA were selected for study to provide a range in GC content. To assess cytokine production, we initially focused on IL-12 since previous studies have shown that this cytokine provides an early and sensitive index of macrophage stimulation (16, 17). Figure 1 shows dose response curves of the DNA for *in vitro* stimulation of IL-12. As these data indicate, bacterial DNA, but not mammalian DNA, can stimulate IL-12 production, although the magnitude of the response varies among the DNA. In the DNA panel tested, EC and MC DNA induced the highest level of IL-12 production. CP DNA was least active among the bacterial DNA although it did induce measurable amounts of IL-12 at the highest concentration tested (50 $\mu\text{g/ml}$).

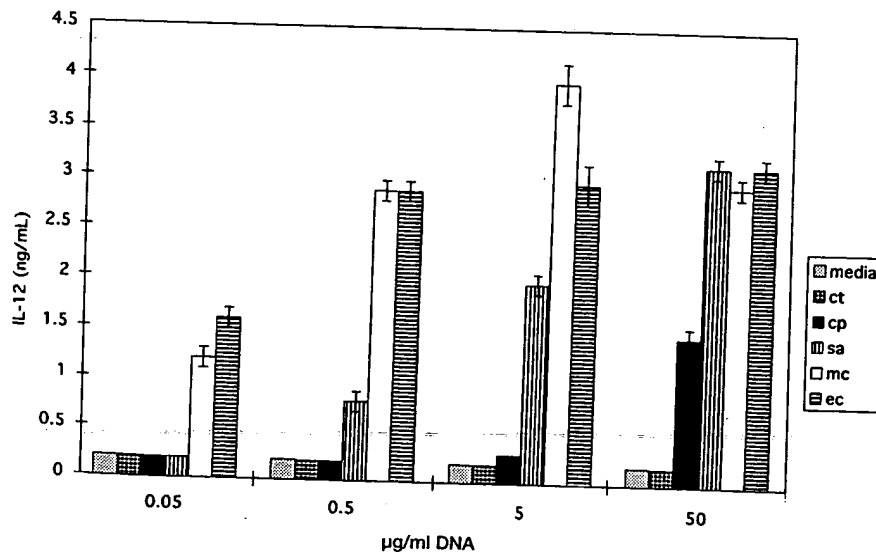


Figure 1. Dose response for IL-12 p40 production. BALB/c spleen cell cultures were stimulated for 24 hours with bacterial DNA at various concentrations. IL-12 was measured in supernatants by ELISA. Results are presented as mean values \pm SD of triplicate wells. Data are from a representative experiment which was performed three times.

Table 1. Effect of DNase on cytokine stimulation by bacterial DNA.

Species of DNA	BALB/c		C3H/HeJ	
	Intact DNA	Digested DNA	Intact DNA	Digested DNA
CP	≤0.1 ng/ml	≤0.1 ng/ml	≤0.1 ng/ml	≤0.1 ng/ml
EC	3.0 ± 0.5 ng/ml	≤0.1 ng/ml	3.0 ± 0.1 ng/ml	≤0.1 ng/ml
MC	2.8 ± 0.5 ng/ml	≤0.1 ng/ml	2.9 ± 0.1 ng/ml	≤0.1 ng/ml
SA	1.1 ± 0.2 ng/ml	≤0.1 ng/ml	2.8 ± 0.1 ng/ml	≤0.1 ng/ml
CT	≤0.1 ng/ml	≤0.1 ng/ml	≤0.1 ng/ml	≤0.1 ng/ml

BALB/c or C3H/HeJ spleen cells were stimulated with 5 µg/mL of either intact DNA or DNA digested with DNase for 2 hours at 50 µg/mL. IL-12 production was measured by ELISA. Results are reported as means ± std. error of quadruplicate wells.

To demonstrate that stimulation resulted from DNA rather than a contaminant such as endotoxin, we performed experiments using DNA preparations which had been digested by DNase I. As shown in Table 1, pretreatment of the DNA by DNase I completely abrogated the production of IL-12. Furthermore, the responses of endotoxin-resistant C3H/HeJ mice were similar to those of BALB/c mice, indicating that any endotoxin possibly present in the preparations did not influence the pattern of responsiveness observed.

IFN-γ production was next measured as a probe of differences among bacterial DNA in their immune capacity. Although IL-12 can stimulate IFN-γ production, recent studies suggest that the induction of IFN-γ by bacterial DNA may result from a synergistic interaction of both IL-12 and DNA on NK cells (16, 18). Figure 2 shows IFN-γ production induced by the 4 bacterial DNA. As these data indicate, the DNA differed in their induction of IFN-γ, with EC DNA inducing the highest level of this cytokine while CP DNA induced the lowest activity. Thus, for both IL-12 and IFN-γ production, a similar rank order (EC > MC > SA > CP > CT) in DNA potency was observed.

Stimulation of B cells by bacterial DNA

We next assessed the capacity of the different bacterial DNA to stimulate B cell mitogenesis. Although it has been assumed that immune activation of B cells and macrophages involves similar DNA structures, a direct comparison of the activity of natural DNA has not been previously performed. Furthermore, uptake of DNA into macrophages and B cells may differ because of the expression by macrophages of receptors (e.g., Type A MSR) that can bind certain DNA (19, 20). The influence of other DNA sequences on immune activity may therefore become apparent by this comparison.

To test whether bacterial DNA vary in mitogenic capacity, we used two different measures of activation. As shown in Figure 3, the bacterial DNA in the panel differed in their ability to induce thymidine incorporation with EC and CP DNA having the highest and lowest activity respectively. Similar to cytokine

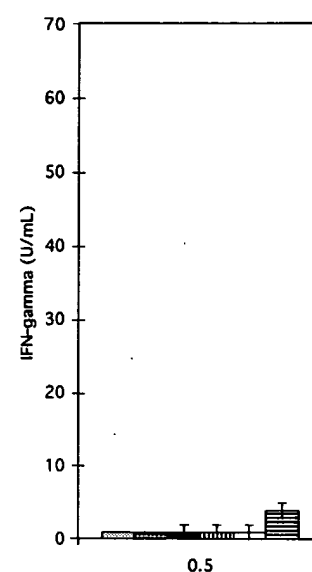


Figure 2. Dose response for IFN-γ of bacterial DNA for 48 hours. E. mean values ± SD of triplicate wells formed three times.

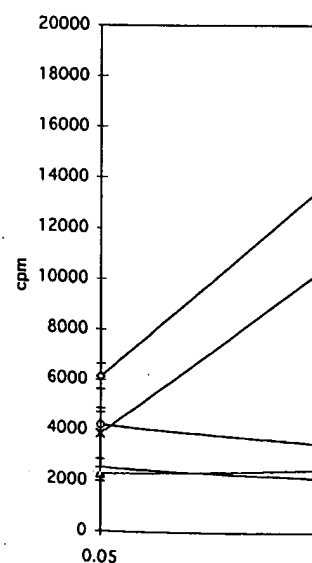


Figure 3. Stimulation of mitogenesis by bacterial DNA for 48 hours followed by measurement of thymidine incorporation. E. mean values ± SD for cultures with representative data from one experiment.

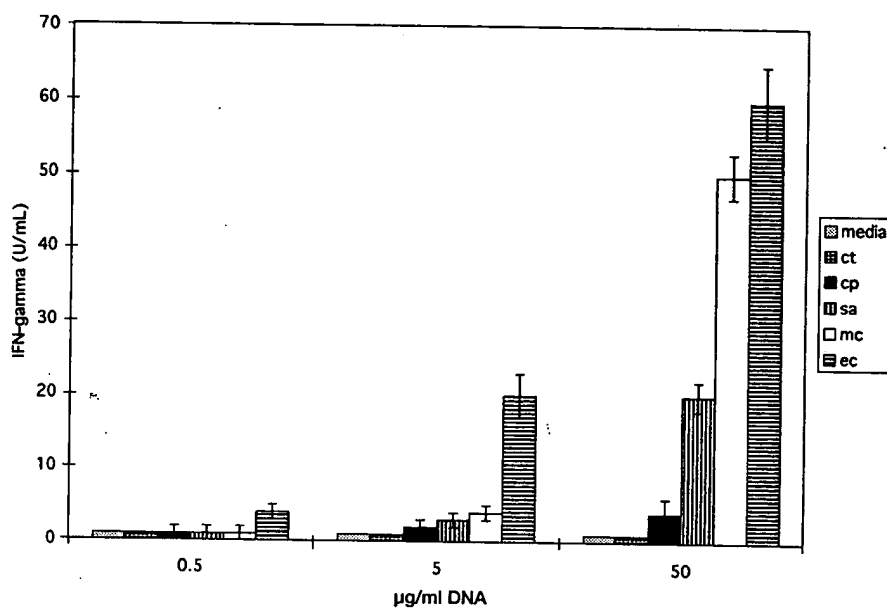


Figure 2. Dose response for IFN- γ production. Spleen cell cultures were stimulated by a panel of bacterial DNA for 48 hours. ELISA for IFN- γ was performed on supernatants. Results are mean values \pm SD of triplicate wells. Data are from a representative experiment which was performed three times.

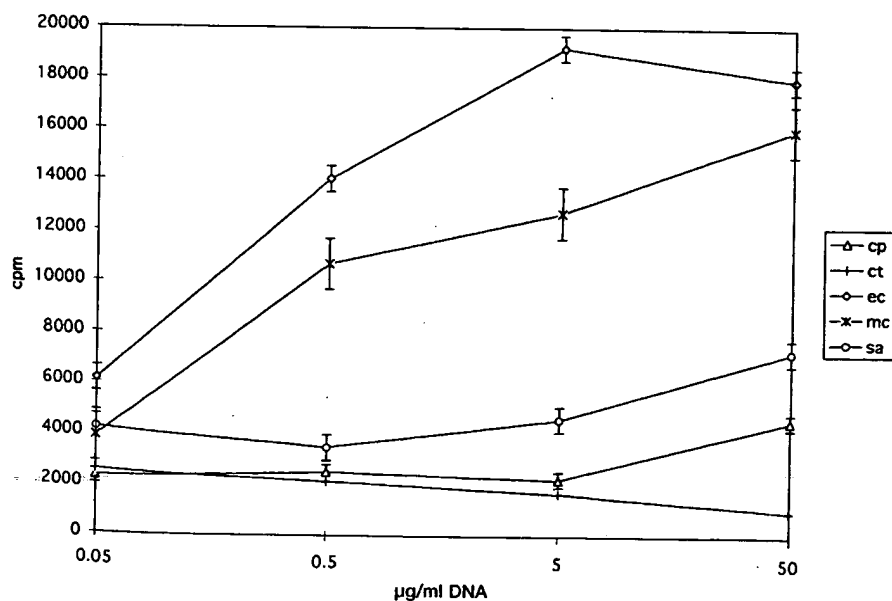


Figure 3. Stimulation of mitogenesis by bacterial DNA. Spleen cells were stimulated with bacterial DNA for 48 hours followed by labeling with ^3H -thymidine for 7 hours. Results are presented as mean cpm \pm SD for cultures in triplicate. The experiment was performed three times with representative data from one experiment presented.

Table 2. Induction of CD69 expression by bacterial DNA.

Species of DNA	0.5 µg/ml	5 µg/ml
CP	9	11
EC	44	54
MC	29	41
SA	17	22
CT	12	12
Media	10	NA
LPS	111	122

BALB/c spleen cells were stimulated by bacterial DNA or LPS at the concentrations indicated. As a measure for mitogenesis, CD69 expression on CD19⁺ cells was determined by FACS. Results are reported as mean fluorescence. The experiment was repeated 3 times with similar results.

production, the stimulation of mitogenesis by bacterial DNA was fully sensitive to DNase (data not shown). Furthermore, endotoxin resistant C3H/HeJ splenocytes showed the same pattern in response to bacterial DNA, confirming that mitogenesis reflects the activity of DNA rather than any endotoxin contamination.

As an additional measure of mitogenesis, we measured by the expression of CD69 by CD19⁺ splenocytes. CD69 is a non-specific marker of immune cell activation and, as shown by SUN et al. (10), is induced by bacterial DNA. The CD69 marker provides an useful adjunct for studying mitogenesis since assessment of thymidine incorporation can be influenced by pool effects resulting from DNA breakdown (15). Theoretically, differences in thymidine content among DNA could affect proliferation assays because of variable effects on pool size. An assay CD69 expression therefore provides an independent measure of cell activation to confirm results of thymidine incorporation.

Table 2 shows the mean fluorescence values obtained after staining cell following 24 hours of *in vitro* stimulation. As these data indicate, in the assay of CD69 expression, the bacterial DNA show the same relative potency observed in assay of thymidine incorporation. Similar to results with thymidine incorporation, EC DNA induced the highest CD69 levels while CP DNA caused the lowest level of induction. These results demonstrate, using two assays of activation, that bacterial DNA differ in their capacity to stimulate B cell mitogenesis.

The effect of lipofectin on immune stimulation by bacterial DNA

At least two possibilities can explain the differences among bacterial DNA in their stimulation of immune responses. The first relates to their content of immunostimulatory sequences. The second relates to their content of other sequences that could promote binding and uptake into cells. Earlier observations have shown that the presence of dG runs in juxtaposition to an ISS can dramatically enhance induction of cytokines, presumably because of binding to the

Type A MSR (11). These observations suggest the presence of sequences affecting the bacterial DNA.

To determine whether the extent of uptake, we assessed to lipofectin. This cationic lipophilic propyl-N,N-triethylammonium amine (DOPE), can bind DNA and induce endocytosis (21). Lipofectin a conditions in which ordinary uptake of these compounds into cells to enhance uptake of DNA into cells, lipofectin is a measure of immune stimulation.

To assess the influence of cytokine production and mitogenesis (at maximal concentration), 50 µg/ml employed DNA/lipofectin at 100 µg/ml indicates that this is an effective concentration. Figures 4–6 present the results of the effect of lipofectin varied amount of DNA on cytokine production caused by lipofectin either IL-12 production or B

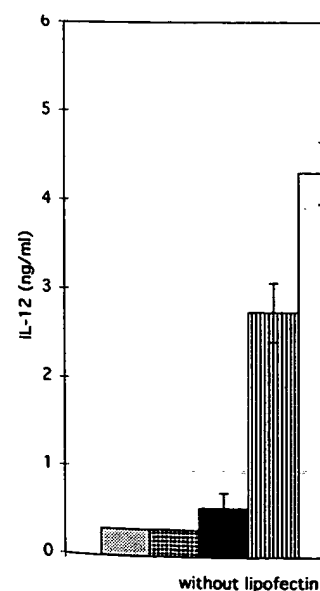


Figure 4. The effect of lipofectin on cytokine production with bacterial DNA (5 µg/ml) without lipofectin was assessed by ELISA of supernatant. Data are mean values ± SD of triplicate cultures.

Type A MSR (11). These observations raise the possibility that differences in the content of sequences affecting uptake could contribute to the relative potency of the bacterial DNA.

To determine whether the activity of bacterial DNA can be influenced by the extent of uptake, we assessed the induction of responses using DNA complexed to lipofectin. This cationic liposome preparation, an 1:1 mixture of dioleoyloxypropyl-N,N-triethylammonium (DOTMA) with dioleylphosphatidylethanolamine (DOPE), can bind DNA and dramatically increase cellular uptake by endocytosis (21). Lipofectin allows immune cell activation by oligos under conditions in which ordinary uptake mechanism fail to deliver adequate amounts of these compounds into cells to induce stimulation (22). By promoting efficient uptake of DNA into cells, lipofectin may provide a more direct and accurate measure of immune stimulation.

To assess the influence of lipofectin on immune stimulation, we compared cytokine production and mitogenesis stimulated by DNA at 5 µg/ml, (a submaximal concentration), 50 µg/ml and 5 µg/ml in the presence of lipofectin. We employed DNA/lipofectin at a weight ratio 1:2 on the basis of preliminary studies indicating that this is an optimal ratio for enhancing cytokine production. Figures 4–6 present the results of these experiments. As these data indicate, the effect of lipofectin varied among the responses. Thus, the increase in IFN-γ production caused by lipofectin was proportionately greater than the increase in either IL-12 production or B cell proliferation. Despite the differences among

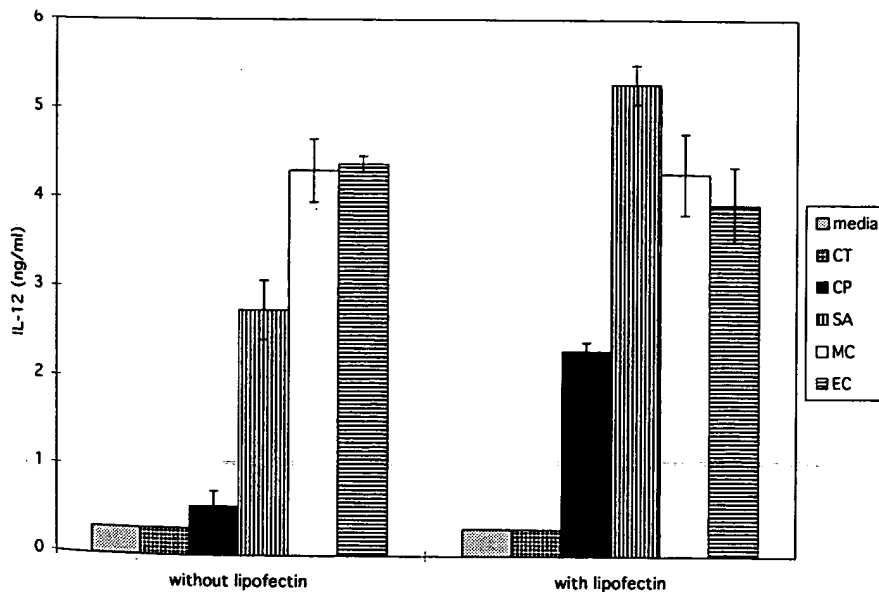


Figure 4. The effect of lipofectin on IL-12 p40 production. Spleen cell cultures were stimulated with bacterial DNA (5 µg/ml) with and without lipofectin (10 µg/ml). IL-12 p40 production was assessed by ELISA of supernatants collected after 24 hours of stimulation. Results shown are mean values \pm SD of triplicate cultures.

Cell Line	50 µg/mL	5 µg/mL	5 µg/mL + lipofectin
CT	16	18	31
CP	18	21	41
SA	33	26	71
MC	29	23	78
EC	43	42	88

responses in the effect of lipofectamine on the DNA with respect to immunogenicity suggest that the relative potency correlates with cytotoxic capacity rather than differences in immunogenicity.

Discussion

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content of ISS.

Studies of synthetic oligos have shown that unmethylated CpG dinucleotides are immunostimulatory. This sequence was originally identified by mycobacterial interferon γ by mycobacterial immunostimulatory activity (2). CpG dinucleotides are an alternate DNA structure called CpG islands. CpG residues to base pair with each other. CpG arrays that can form in either prokaryotes or eukaryotes alone can stimulate B cell activation and antibody production (14). The presence of CpG motifs is important for CpG motif because of the immunostimulatory activity (11). This receptor can bind a variety of oligonucleotides and subsequent

The assessment of the optimum length of the oligonucleotide is complicated by the use of phosphodiester oligos, the substitution of a sulfur atom for phosphorus in the phosphodiester backbone. The use of phosphorothioate oligos increases resistance to nucleolytic degradation and other physical and chemical processes that stimulate immune responses (27). Phosphodiester oligos fail to stimulate immune responses, but phosphorothioate oligos are highly effective in stimulating T cells (28). Furthermore, stimulation of T cells may not directly relate to the chemical nature of the backbone as well as the sequence of the oligonucleotide. Although a variety of structural features have been studied, the results suggest the importance of the sequence of the oligonucleotide in stimulating T cells.

responses in the effect of lipofectin, this agent did not change the rank order of the DNA with respect to immune activation. Taken together, these findings suggest that the relative potency of bacterial DNA reflects their intrinsic stimulatory capacity rather than differences in binding and uptake.

Discussion

The work presented herein demonstrates marked differences among bacterial DNA in their *in vitro* immunostimulatory activity. Thus, among a panel of bacterial DNA, EC DNA was the most potent stimulator of B cell mitogenesis as well as cytokine production while CP DNA was the least potent. These differences were observed even in cultures in which DNA was introduced in the presence of lipofectin to overcome any potential differences in the extent of DNA binding and uptake. Taken in concert with previous observations on the influence of DNA base sequences on IFN- α/β production (23), these findings suggest a wide variation in immune capacity of bacterial DNA that is related to the content of ISS.

Studies of synthetic oligos have identified a variety of sequence motifs that cause immunostimulation. The most prominent of these sequences consists of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines. This sequence was originally identified in a study of the stimulation of interferon by mycobacterial DNA, and subsequently shown to have broad immunostimulatory activity (2, 3, 24). Another sequence that can contribute to immunostimulation is an extended run of dG residues. dG runs can form an alternate DNA structure called quadruplex DNA because of the ability of dG residues to base pair with each other. Quadruplex DNA consists of four strand arrays that can form in either parallel or an parallel orientation (12, 25). dG runs alone can stimulate B cell activation but do not cause macrophage cytokine production (14). The presence of dG runs, however, can increase the activity of a CpG motif because of the interaction of G-rich DNA with the Type A MSR (11). This receptor can bind a variety of polyanions and can increase uptake of oligonucleotides and subsequent activation of macrophages.

The assessment of the optimal ISS for B cells as well as macrophages has been complicated by the use of phosphorothioate derivatives. These compounds have the substitution of a sulfur atom for one of the non-bridging oxygens in the phosphodiester backbone. The presence of this substitution can increase nuclease resistance and other physical properties of DNA, including a capacity to stimulate immune responses (26, 27). Thus, while bacterial DNA and CpG phosphodiester oligos fail to stimulate human B cell activation, phosphorothioate oligos are highly effective and can activate these cells in the absence of T cells (28). Furthermore, stimulation of B cell responses by phosphorothioates may not directly relate to the content of CpG motifs, suggesting the contribution of the backbone as well as sequence (26).

Although a variety of structural features may lead to immune stimulation, our results suggest the importance of CpG motifs in the responses induced by bacte-

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rial DNA. Among the DNA we studied, EC DNA, which was the most active, has a GC content of 50% while the least active DNA, CP DNA, has a GC content of 27%. Furthermore, in a previous study on the stimulation of IFN- α/β by DNA from various species, the frequency of potent hexameric sequences in these genomes was calculated (23). While some of these sequences did not display the purine-prime-CpG-pyrimidine-pyrimidine motif, all contained CpG and were palindromic. On the basis of GenBank data, EC DNA contains 2.4 potent hexameric CpG motifs per 1000 bases compared with only 0.2 in the CP genome.

The concentration of potent hexamer CpG motifs may not be the sole determinant of immunostimulatory potential, however. Thus, among the DNA we studied, MC DNA shows 4.4 potent hexamers per 1000 bases, although this DNA was less active than EC DNA. Furthermore, a study by SUN et al. (10) indicated that unmethylated mammalian DNA lacks immunostimulatory activity although this DNA would be predicted to have ISS. Finally, BALLAS et al. showed that certain 15-mer oligos that contained CpG but lacked a palindromic structure could increase NK cell activity (18). These findings suggest a number of possibilities: 1) the context of ISS (i.e., their distribution in the genome or the presence of surrounding sequences) may influence their activity; 2) certain DNA, either mammalian or bacterial, may contain inhibitory sequences that block stimulation; and 3) DNA may differ in their entrance into cells, with the level of immunostimulation reflective of the concentration of intracellular DNA as well as the number of ISS.

To determine whether differences in cell binding and uptake could explain any of the differences in stimulatory capacity observed, we assessed the activity of DNA in the presence of lipofectin. Lipofectin allows DNA to enter cells by non-receptor mediated endocytosis (21). Once inside cells, lipofectin facilitates DNA passage into the cytoplasm, most likely because of its destabilizing effects on endosomal membranes (29). In the presence of lipofectin, oligos as short as six bases can induce IFN- γ production as long as they contain the sequences ACGT, although these oligos are inactive alone (30). These findings suggest that the use of lipofectin may provide a better assessment of immunostimulation since this agent can overcome limitations in cellular entry of DNA and increase intracellular concentration to levels sufficient for stimulation. Although the effect of lipofectin on DNA uptake is likely similar for all natural DNA, we are directly assessing this issue using fluorescently-labeled DNA.

When introduced into cultures with lipofectin, all DNA showed an increase in activity, although the extent varied depending on the response measured. Thus, the increases in IFN- γ activity were proportionately greater than increases in either IL-12 production or B cell mitogenesis. In a study of the effect of lipofectin on responses induced by plasmid vectors, we also observed that the presence of lipofectin induces far greater increases in IFN- γ than IL-12 production; in this system, the IFN- γ response is nevertheless dependent of IL-12 (unpublished data). To account for this finding, we have suggested that lipofectin causes an increase of DNA uptake into IFN- γ producing NK cells that is greater than the increase in either macrophage or B cells. As a result, the IFN- γ response

shows a greater increase from lipofectin. Macrophages are unable to respond to bacterial DNA ergistically to stimulate IFN- γ by the augmentation observed (16, 18).

While the mechanism of the lipofectin nevertheless suggest that differences in DNA do not explain for the relative potency, contrasts with studies on synthetic oligonucleotides that allow receptor mediated stimulation (11). On the other hand, we have found that the ratio of dG to plasmids does not increase stimulation. These findings suggest the mechanisms of immunostimulation by plasmids may differ, with oligonucleotides having a higher molecular weight DNA from plasmids.

Taken together with other findings, the induction of immune responses by the content of ISS and their distribution differences could impact on host/pathogen immunity by various pathogenic species. These actions are the organism's array of immunostimulatory molecules, including endotoxin, lipoteichoic acid and DNA (31) termed «danger signals.»

In the setting of infection, danger signals stimulate innate immunity, with resulting release of these molecules. As our results indicate, the activity and resembles endotoxin in its stimulation of both endotoxin and bacterial immunity. It is important to inquire whether the outcome of danger molecules displays a direct effect on the progress to elucidate the stimulation and the mechanisms by which this ultimately affects immune responses.

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shows a greater increase from lipofectin than IL-12. Although NK cells alone are unable to respond to bacterial DNA, bacterial DNA and IL-12 may act synergistically to stimulate IFN- γ by these cells, leading to the disproportionate augmentation observed (16, 18).

While the mechanism of the lipofectin action is not clear, these experiments nevertheless suggest that differences in binding and uptake of DNA into cells do not explain for the relative potency of the bacterial DNA. This finding contrasts with studies on synthetic oligonucleotides which demonstrate that macrophage stimulation by an ISS can be dramatically altered by sequences (e.g., dG runs) that allow receptor mediated uptake as opposed to fluid-phase endocytosis (11). On the other hand, we have shown that the addition of extended runs of dG to plasmids does not increase stimulation of IL-12 and IFN- γ (20). These findings suggest the mechanisms of immune stimulation by oligonucleotides and plasmids may differ, with oligonucleotides more affected by sequence changes than high molecular weight DNA from either plasmid or bacterial genomes.

Taken together with other findings, our results point to striking differences in the induction of immune responses by bacterial DNA that most likely relate to the content of ISS and their distribution among other genomic sequences. These differences could impact on host/pathogen interactions and induction of innate immunity by various pathogenic species. Among factors influencing these interactions are the organism's array of immunostimulatory molecules such as endotoxin, lipoteichoic acid and DNA (31). These molecules have collectively been termed «danger signals.»

In the setting of infection, danger signals may act alone or in concert to stimulate innate immunity, with resulting responses reflecting the composite action of these molecules. As our results indicate, bacterial DNA does not have uniform activity and resembles endotoxin in its range of potency (32). In view of variation of both endotoxin and bacterial DNA in signaling danger, it will be important to inquire whether the outcome of infection depends on the number and potency of danger molecules displayed by bacterial species. Studies are in progress to elucidate the stimulation of different cell types by bacterial DNA and the mechanisms by which this ubiquitous macromolecule elicits protective immune responses.

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